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CHARACTERIZATION OF OLFACTORY RECEPTOR NEURONS AND OTHER CELL TYPES IN DISSOCIATED RAT OLFACTORY CELL CULTURES

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Abstract—In dissociated cell cultures, control over the cellular environment facilitates study of the differentiation of mature cellular phenotypes. Central to this approach is a rigorous characterization of the cells that reside in culture. Therefore, we have used a battery of cell type-specific antibody markers to identify the cell types present in dissociated cultures of olfactory mucosal cells (containing cells from both the epithelium and lamina propria). To identify olfactory receptor neurons in the cultures, staining with antibodies against neuron-specific tubulin was compared to staining with antibodies to neuron-specific enolase, the neural cell adhesion molecule, N-CAM, and the adhesion molecule, L1. Staining of mature olfactory neurons in culture, with an antibody against the olfactory marker protein, was compared to staining with antibodies to carnosine. In contrast to tissue section staining, the overlap between carnosine and olfactory marker protein staining was not complete. Olfactory nerve glial cells were immunoreactive for the S100 β protein and nestin, an intermediate filament found in early neuronal progenitor cells and Schwann cells. Antibodies to nestin did not label olfactory neurons or progenitor cells. An antibody to an oligodendrocyte-Schwann cell enzyme, 2',3'-cyclic nucleotide 3'-phosphodiesterase, did not label olfactory glia, but did label oligodendrocyte-like cells that appeared to be derived from the CNS glial feeder layer. An antibody against the heavy (200 kDa) neurofilament protein stained a minor subset of cells. The cultures also contained muscle cells, cartilage cells and macrophages (and/or microglia). These results demonstrate that multiple cell types either maintain or re-establish differentiated, cell type-specific phenotypes in dissociated olfactory cell cultures. Copyright © 1996 ISDN.

Key words: olfactory mucosa, immunocytochemistry, olfactory marker protein, carnosine, neural cell adhesion molecules, neuron-specific enolase, neuron-specific tubulin, neurofilaments, nestin, muscle, cartilage

INTRODUCTION

The olfactory neuroepithelium is a specialized region of the lining epithelium of the nasal cavity. It is a pseudostratified columnar epithelium that contains the cell bodies of the olfactory receptor neurons (ORNs). These neurons extend their dendrites to the apical surface of the epithelium, where their dendritic knobs and sensory cilia sit in the airspace of the nose, covered by layers of mucus. These are the only neurons that have cellular processes that contact the external environment. Perhaps because of this, the olfactory neuroepithelium has the capacity to undergo more extensive neurogenesis throughout life than any other nervous tissue.^{13,18,19} Developing an understanding of this capacity for neurogenesis could allow us to determine why other nervous tissues do not have similar regenerative capabilities.

In preparation for studies of olfactory neurogenesis, our laboratory has developed cell cultures that support production of ORNs. We demonstrated previously that the neurons produced in this culture system are able to undergo multiple phases of neuronal differentiation.⁴² These phases include an initial phase in which the neurons express proteins that are also found in other types of neurons and a second, later phase in which they express a protein found almost exclusively in ORNs, the olfactory marker protein (OMP).⁴²

Expression of specific proteins by different cell types can be a valuable experimental tool for researchers. This allows cell-type identification and further study of cell biology, both *in vivo* and in cell culture.

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In developing cell cultures containing ORNs, we found that simple systems in which cells from olfactory tissues were plated at low density did not support *in vitro* neurogenesis.⁴⁹ Instead, it was necessary to plate cells at high density on a feeder layer of central nervous system (CNS) glial cells (astrocytes).⁴² Under these conditions, neurons are generated and differentiate. In addition, they form complex aggregates with an unusual internal organization. This internal organization includes formation of central, fluid-filled cavities. The neurons are located primarily at the exterior of the aggregates and they have a very specific orientation; most extend their dendrites towards the central cavities. These aggregates also contain several of the supporting cells of the olfactory epithelium, including sustentacular cells and Bowman's gland cells^{47,48} (also Pixley, in preparation). Because of these and other similarities to the olfactory epithelium *in vivo*, we have termed these aggregates, micro-noses.⁴⁶

As just described, we found it necessary to generate very complex cultures, with extensive cellular heterogeneity, in order to obtain ORN generation and differentiation. Thus, cellular heterogeneity appears to be advantageous. Another advantage of this complexity is that it allows us to study cell-cell interactions that may be crucial to ORN biology. However, these interactions can only be studied if the heterogeneity is well defined, e.g. if we know which cell types are present and where they are in relationship to the ORNs. Towards this end we have further characterized the cell types in our olfactory cell cultures.

The tissues that are taken from the animal to produce ORN cultures include all of the soft mucosal tissue that sits on the cartilagenous turbinates and midline septum in the posterior regions of the nasal cavity. In the rat, the turbinates and midline septum are covered predominantly by olfactory (not respiratory) epithelium. The soft mucosal tissue includes both the olfactory epithelium itself and the underlying connective tissue lamina propria. The olfactory epithelium contains essentially four cell types: the ORNs, supporting cells called sustentacular cells and two types of basal cell that do not have processes extending from the basal lamina to the apical surface. The basal cell types include a keratin-positive, flat basal cell and a round, highly mitotic globose basal cell. Also tightly associated with the epithelium are the Bowman's glands, serous submucosal glands that extend frequent ducts through the epithelium to the nasal cavity. The acini of the glands lie in the loose connective tissue of the lamina propria. The lamina propria also contains blood vessels and the olfactory nerves, which are bundles of ORN axons surrounded by the olfactory nerve glia and a fibroblastic endoneurium and perineurium.

In this report, we use a panel of cell type-specific antibodies to identify cell types. We describe immunostaining with several neuronal markers and markers to multiple other cell types present in the lamina propria. These studies are one part of a multi-faceted characterization of the cellular make-up of the cultures. They set the stage for more complex analyses of regulatory factors acting on neuronal generation and differentiation.

EXPERIMENTAL PROCEDURES

Cell culture

Methods for cell culture were as described previously^{21,42,43,46} with slight modifications, indicated below. All cells were prepared from neonatal (postnatal day 0-3) Sprague-Dawley rats. Briefly, after cold-induced anesthesia and decapitation, the structures taken from the animals were the cribriform plate with the attached midline septum and turbinates. The midline septum rostral to the turbinates was discarded to decrease the possibility of respiratory tissue contamination. The cartilage and overlying soft tissues were placed in a sterile balanced salt solution lacking Mg^{2+} and Ca^{2+} and minced into pieces small enough to fit through the tip of a 10 ml plastic pipette. This material was rinsed three times with fresh solution and then dissociated by enzymatic (trypsin) and mechanical means to produce a dissociated cell suspension. At this point, the cell suspension was passed through a 210 μm nylon mesh sieve to remove cartilage. The cells that passed through the sieve were collected, rinsed further by centrifugation, counted, then diluted and plated in a serum-free growth medium (see below).

The serum-free growth medium for the cell cultures, designated as DSN1,²¹ consisted of the basal medium, Dulbecco's modified eagle's medium with 4.5 g/l glucose and 3.7 g/l sodium bicarbonate,

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with added 18 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 1 × modified Eagle's medium non-essential amino acid solution (g/l: 0.89 L-alanine-HCl, 1.5 L-asparagine-H₂O, 1.33 L-aspartic acid, 1.47 L-glutamic acid, 0.75 glycine, 1.15 L-proline, 1.05 L-serine, purchased as Sigma No. M-7145), 4 mM glutamine, 30 μM hypoxanthine, 3 μM thymidine, 100 Units/ml penicillin, 0.1 mg/ml streptomycin, 57 μM 2-mercaptoethanol (we now recommend 28.5 μM),²¹ and a modification of the N1 supplements of Bottenstein⁴ (10 μg/ml transferrin, 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine and 30 nM sodium selenite).

The olfactory cell suspension was plated onto a feeder layer of CNS cortical astrocytes. We will call this combined preparation an "olfactory cell" culture because of the presence of olfactory epithelial cells. However, this preparation contains both olfactory epithelial cells and cells from the connective tissue lamina propria underneath the epithelium. The astrocytes were prepared 2–4 weeks prior to plating nasal cells, by dissociation of cortical tissues from neonatal rat pups, as described previously.^{42,43}

One modification of the astrocyte feeder layer preparation was used in these studies. In earlier studies, the cortical cell suspension prepared from the newborn pups was initially plated at 2×10^5 cells/ml (10 ml/T75 flask). When the cells were ready to be passaged to provide feeder layers, at 7–10 days *in vitro*, the cultures were cellularly heterogeneous. A confluent layer of flat astrocytes was surmounted by small cells that were dark in phase contrast optics (phase-dark cells). The phase-dark cells are a mixture of oligodendrocytes, microglia, glial progenitor cells and occasional neurons.²⁷ Overnight shaking was needed to eliminate the loosely attached phase-dark cells before taking the underlying astrocytes (with trypsin and EDTA) and re-plating them as a feeder layer. We found that if the cortical cells were instead plated initially at a 10-fold lower concentration (2×10^4 cells/ml), the resultant cultures did not have phase-dark cells. This eliminated the need for shaking and produced a more homogeneous feeder layer. In either case, both preparations contained over 90% positive cells when stained with glial fibrillary acidic protein (GFAP) antibodies (indicating CNS astrocytes) and both supported abundant survival and production of OMP-immunoreactive (OMP-IR) cells when used as feeder layers.

After plating olfactory cells on astrocyte feeder layers, the cells were maintained in serum-free medium as previously described^{21,42} for 15 days then fixed with 4% paraformaldehyde (15 min, room temp) and immunostained.

Different cell cultures were used for staining with antibodies to neuron-specific enolase (NSE), in order to demonstrate better the morphological differences between ORNs and olfactory glial cells. For anti-NSE staining, olfactory cells were plated on polylysine-coated glass coverslips without an astrocyte feeder layer and grown for only 5 days *in vitro*, as described previously.^{21,42} A different fixative was also used for NSE staining to avoid washing out the highly soluble NSE. The cells were fixed with 4% paraformaldehyde, 0.5% glutaraldehyde, 0.2% saturated picric acid, in 0.1 M sodium acetate buffer, pH 6.0, before immunostaining.²¹

Immunocytochemistry

Primary antibodies, dilutions and sources are given in Table 1. Methods for immunostaining have been described previously.^{21,42,49} Briefly, non-specific binding was blocked with a 1 hr incubation in a balanced salt solution containing 0.2% Triton, 10% horse serum and sodium azide. Dilutions of primary and secondary antibodies were in phosphate-buffered saline (PBS) with 0.2% Triton and rinses were with PBS. Primary antibody incubations were overnight at room temperature and secondary and tertiary reagents were incubated for 2 hr each, at room temperature. Control normal rabbit serum (NRS) was from Chemicon (Temecula, CA, U.S.A.). Pre-absorption with OMP was done by incubating anti-OMP with purified recombinant rat OMP (a generous gift from F. Margolis) for 24 hr prior to staining. The OMP protein concentration was 1 μM in the final staining volume with a 1/30 000 dilution of anti-OMP.

For bright field examination, peroxidase labeling was done. The secondary antibodies were species-specific biotinylated antibodies from Vector Labs (Burlingame, CA, U.S.A., each at 1/500 dilution), followed by incubation in the avidin-biotin-horseradish peroxidase "Elite ABC" reagent from Vector Labs (Burlingame, CA, U.S.A., 1/500 dilution). Color was developed using diaminobenzidine (0.5 mg/ml) and 0.03% H₂O₂.

For fluorescent labeling, the secondary antibodies were FITC-labeled donkey anti-goat IgG (at

Table 1. Antigens, animal origin, dilutions and sources for the antibodies used in this study

Antigen	Antibody	Dilutions	Use*	Source of antibody
Neuron-specific tubulin (NST) (class β , isotype III) (TUJ1)	Mouse monoclonal (TUJ1)	1/10 000	B	A. Frankfurter
Neuron-specific tubulin (NST) (class β , isotype III)	Mouse monoclonal	1/5000	B	Sigma, St Louis, MO, U.S.A.
N-CAM	Mouse monoclonal (N-CAM-OB11)	1/50, 1/100, 1/500	IP	Sigma, St Louis, MO, U.S.A.
L1	Rabbit polyclonal	1/3000	B	V. Lemmon
Neuron-specific enolase (NSE)	Rabbit polyclonal	1/5000, 1/10 000	IP	Polysciences, Inc., Warrington, PA, U.S.A.
Olfactory marker protein (OMP)	Goat polyclonal	1/30 000	B	F. Margolis
Carnosine	Rabbit polyclonal	1/1000	B	F. Margolis
S100 β Protein	Rabbit polyclonal	1/5000	B	Dakopatts, Denmark
Nestin	Rabbit polyclonal	1/2000	IP	R. McKay (sera No. 130)
2',3'-Cyclic nucleotide phosphodiesterase (CNPase)	Rabbit polyclonal	1/8000	IP	T. Sprinkle
200 kDa Neurofilament protein (NF)	Rabbit polyclonal	1/1000	IP	Sigma, St Louis, MO, U.S.A.
Muscle-specific actin (HUC 1-1)	Mouse monoclonal	1/50	IP	J. Lessard
Complement receptor 3 (MRC OX-42)	Mouse monoclonal	1/5000	IP	Serotec, Harlan Bioproducts for Science, Inc., Indianapolis, IN, U.S.A.

*Use: the dilutions given are for immunoperoxidase (IP) or for both (B) immunoperoxidase and immunofluorescence staining.

1/250) and Texas Red-labeled donkey anti-rabbit IgG (at 1/500) (Jackson ImmunoResearch Labs, Inc., West Grove, PA, U.S.A.). Techniques for embedding immunostained culture materials in plastic, thin sectioning and staining with toluidine blue have been described previously.⁴⁶ Photography was done on a Nikon FX microscope with bright field, epifluorescence and differential interference contrast (DIC) optics, with TMAX ASA 400 film for fluorescent staining and TMAX ASA 100 film for bright field staining.

RESULTS

Neuronal staining

NST. Previous studies have demonstrated that antibodies to neuron specific tubulin (class β , isotype III tubulin) (NST) are excellent markers for both immature and mature ORNs in neonatal rat tissue sections²⁵ and in dissociated olfactory cell cultures, where the neurons form spherical aggregates with neuroepithelial walls and fluid-filled central cavities that have been termed micro-noses.^{42,46}

Figure 1(A) shows NST staining (with the monoclonal antibody TUJ1) including one relatively small micro-nose. Similar staining patterns were obtained with a commercial anti-NST antibody from Sigma (St Louis, MO, U.S.A.) (not shown). An advantage of the micro-noses is that the cells within them are primarily derived from the neuroepithelium, while the feeder layer contains primarily cells from the lamina propria. Thus cells could be categorized according to whether or not they were within the micro-noses, like NST-positive neurons, or they were associated with the feeder layer. These cues aided cell type classification.

N-CAM. Antibodies to the neural cell adhesion molecule, N-CAM,^{6,7,23,24,36} and its polysialylated, embryonic form, E-N-CAM,^{34,35} label both immature and mature ORNs in neonatal rodent tissue sections. They also stain olfactory nerve glial cells.^{35,36} The olfactory nerve glia surround and invest the bundles of olfactory nerve axons in the lamina propria. They have been termed the "olfactory ensheathing cells".¹² It has been shown previously that these glia include a large number of Schwann cell-like glia (olfactory Schwann cells) and a subset of more astrocyte-like glia.⁴³

In the olfactory cell cultures that contained micro-noses, a monoclonal antibody to N-CAM

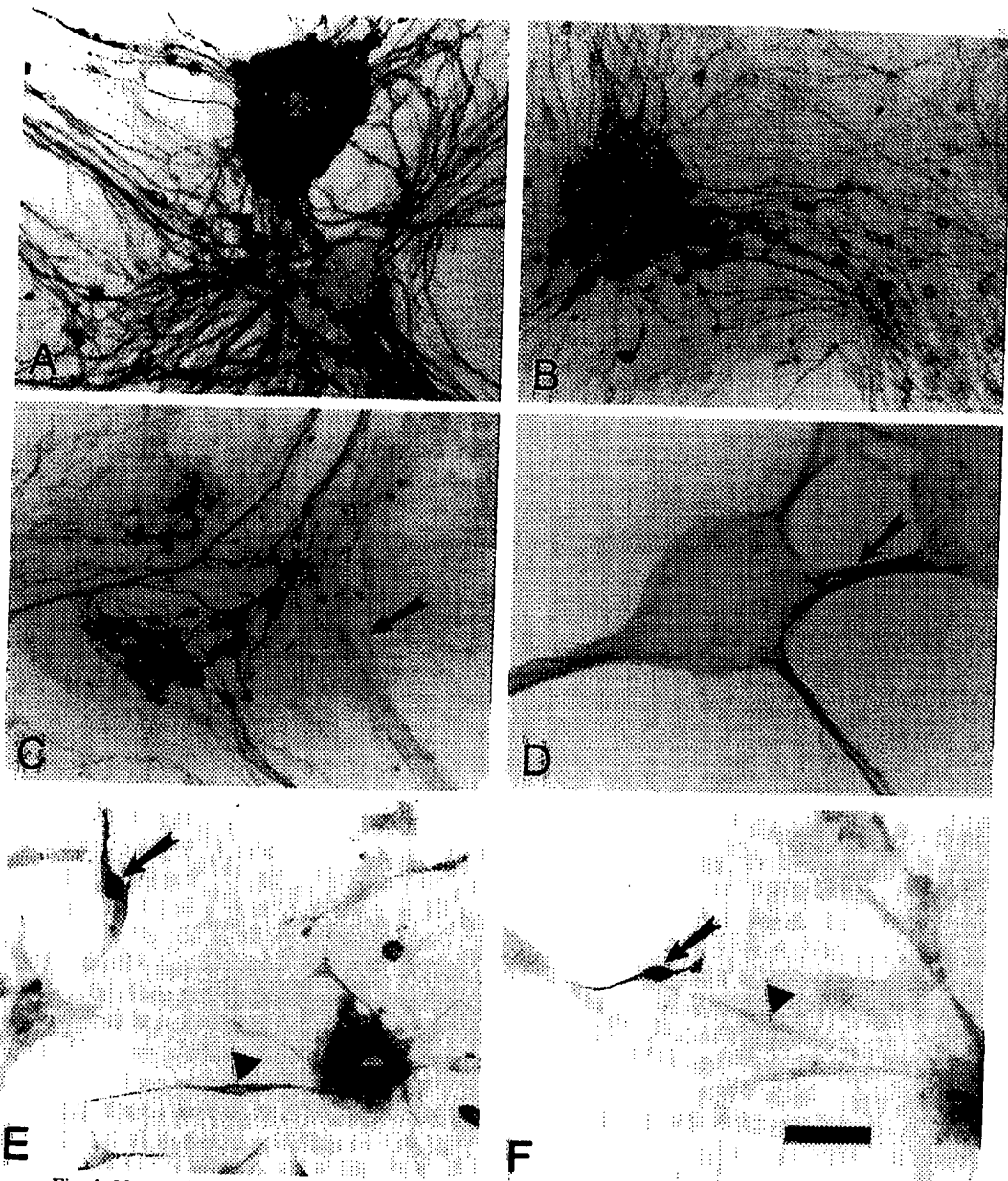


Fig. 1. Neuronal markers. (A) Dense staining of both processes and cell bodies is observed inside and outside micro-noses, when cells were fixed at 15 days *in vitro* and stained with a monoclonal antibody to NST (TUJ1). (B) and (C) Similar staining is seen with anti-N-CAM (1/50 and 1/100 dilution, respectively). The arrow in (C) indicates low level staining of feeder layer cells. (D) Anti-L1 stained primarily cell processes in the neuritic bundles interconnecting the micro-noses (arrow). Little staining was detected within the micro-noses. (E) In low density olfactory cell cultures grown for 5 days without an astrocyte feeder layer, anti-NSE staining at 1/5000 dilution shows dense labeling of neurons (arrow) and glial cells (arrowhead). (F) At a dilution of 1/10000, anti-NSE staining of glial cells (arrow-head) in the same type of culture had almost disappeared, while intense neuronal staining was retained (arrow). Bar = 80 μ m for (A)-(D), 78.8 μ m for (E) and (F).

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intensely labeled a subset of cells at dilutions of 1/50 [Fig. 1(B)], 1/100 [Fig. 1(C)] and 1/500 (not shown). This antibody binds to both adult and embryonic forms of N-CAM (manufacturer's specifications). The stained cells appeared identical to those observed with anti-NST in terms of morphology, number and arrangement within micro-noses. This suggested that the staining was of immature and possibly also mature ORNs. Faint staining of the feeder layer was detected at dilutions of 1/100 [Fig. 1(C), arrows] or 1/50 [Fig. 1(B)]. The faint staining was not examined further.

L1. The ORN axons *in vivo* stain with antibodies to the neural cell adhesion molecule, L1.^{17,36} However, the olfactory nerve glia (ensheathing cells) also stain.³⁶ In our olfactory cell cultures, anti-L1 intensely labeled cell processes in the neurite-containing bundles radiating outward from the micro-noses [Fig. 1(D), arrow]. Fainter staining was detected within the micro-noses, possibly in cell bodies [Fig. 1(D)]. Confocal microscopic analysis of the double-labeled neuronal processes showed areas of overlap and areas of non-overlap with NST-positive fibers (not shown), possibly reflecting both glial and neuronal labeling. However, double labeling with glial markers was not done to confirm or deny glial staining.

Neuron-specific enolase. Neuron-specific enolase is the γ_2 homodimeric form of a glycolytic pathway enolase that appears to be specifically produced by neurons.²⁹ Non-neuronal enolase is the α_2 homodimer, which is found in astrocytes.²⁹ The heterodimeric form of the enolase, $\alpha\gamma$, is not found in brain, but it is found in several cell types outside the nervous system.²⁹ In previous studies, we found that NSE staining could be used to identify ORNs in culture, but only if a special fixative was used and careful attention was paid to the dilution of the antiserum.²¹ The different fixative retains the highly soluble enzyme better than conventional paraformaldehyde fixation.⁵⁸

We demonstrate here some of the problems with NSE staining. Different concentrations of NSE antisera gave roughly three levels of staining intensity in the olfactory cell cultures. To demonstrate the staining, we used low density cultures (olfactory cells plated on polylysine without a feeder layer) because they allow easier visualization of cell morphology compared to staining in the higher density micro-nose cultures. Staining at the highest anti-NSE dilution tested, 1/1500, is not shown because almost all cells were densely stained. The only unstained cells were epithelioid-like cells that formed tightly packed islands of cells. Similar cells were keratin-positive in another study of the same type of culture, which suggests that they are the horizontal basal cells of the olfactory epithelium.⁴⁴ At an anti-NSE dilution of 1/5000, cells with the morphology of neurons were densely stained [arrow in Fig. 1(E)]. Epithelioid cells again were unstained (not shown), but almost all other cells were moderately stained. This included cells that appeared to be olfactory glia or fibroblasts [arrow-head in Fig. 1(E)]. At an anti-NSE dilution of 1/10 000, the glial-like cell staining was reduced or completely eliminated [arrow-head in Fig. 1(F)] while cells that we presumed were neurons remained densely stained [arrow in Fig. 1(F)].

In micro-nose cultures fixed at 15 days *in vitro*, only a few cells were stained with a 1/10 000 dilution of anti-NSE (not shown). These cells were neuronal in morphology but they did not show the same distribution or numbers as cells stained with antibodies to NST, N-CAM or anti-OMP (see below). Some stained cells were located in the micro-noses while others were located on top of the feeder layer cells.

Olfactory marker protein. The OMP-like immunoreactivity (OMP-IR) was detected in a subset of neurons in the micro-noses and in the bundles of processes radiating outward from the micro-noses [Fig. 2(A) and (C); see also Pixley and colleagues^{42,46}]. These are presumed to be older, more differentiated, more "mature" ORNs based on comparisons with staining *in vivo*, and based on tritiated thymidine studies in micro-nose cultures.⁴² The OMP staining was variable in intensity and both cytoplasmic and nuclear [Fig. 2(A); also, see Pixley⁴²]. Only occasional processes were stained. Normal goat serum substituted for the primary antibody gave no staining [Fig. 2(D)]. The OMP-IR was abolished completely by pre-absorption with purified OMP protein [Fig. 2(E)].

Carnosine. In the peripheral olfactory tissues, only ORNs immunostain for the dipeptide carnosine (β -alanyl-L-histidine).^{1,2,40,51-54} In the CNS, particularly in the olfactory bulb and cerebral cortex, carnosine staining also is detected in astrocytes.² In the micro-nose cultures, antibodies to carnosine intensely labeled cells resembling ORNs [Fig. 2(B)]. No flat cells resembling CNS astrocytes were stained at the dilutions used. Labeling was primarily nuclear; cytoplasmic staining was less obvious and process staining was rare. Double labeling with anti-OMP confirmed that at least some of the carnosine-positive cells were also OMP-positive [Fig. 2(A) and (B), asterisks].

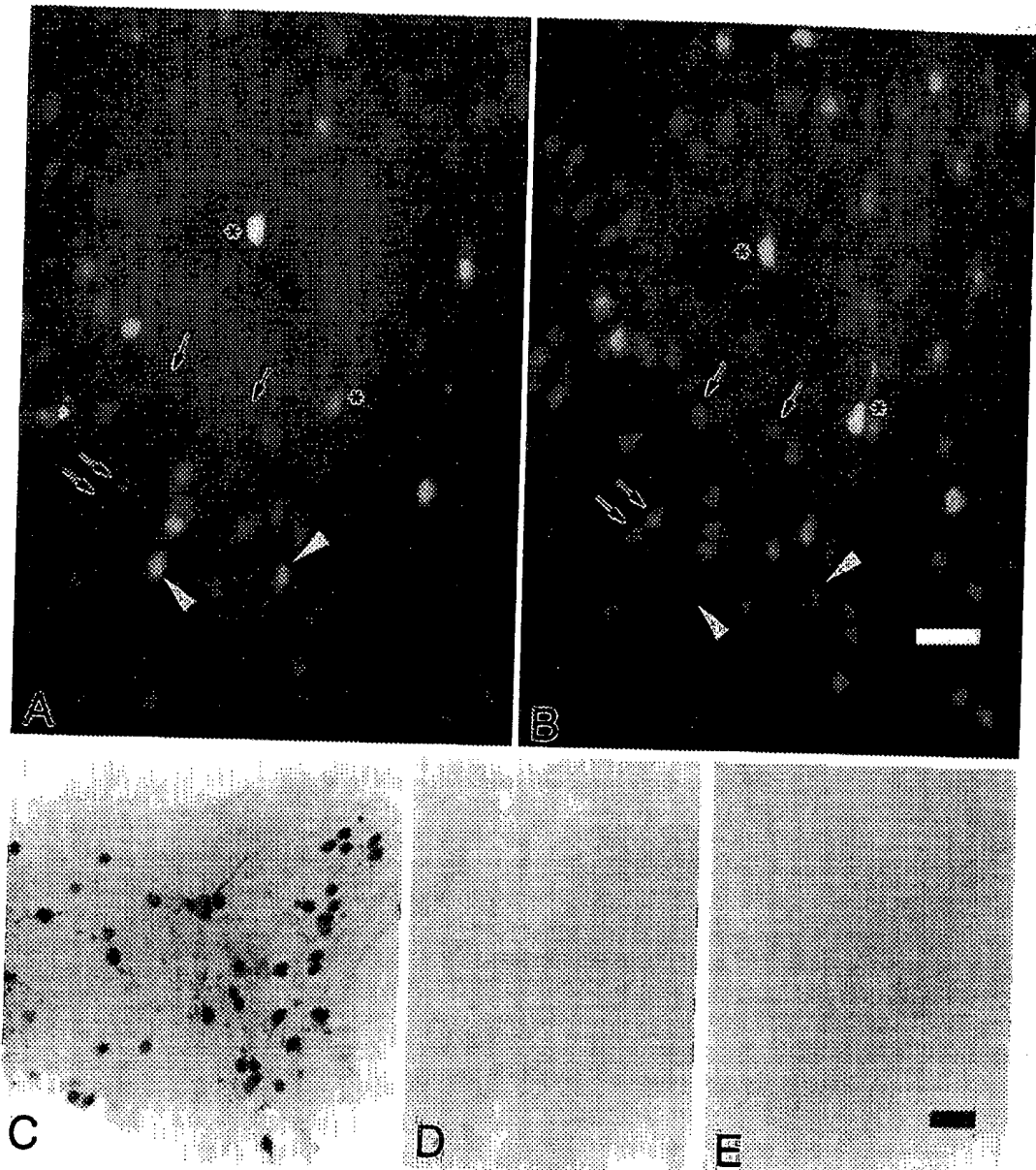


Fig. 2. Olfactory neuron markers, in 15-day micro-nose cultures. Double labeling was done with anti-OMP (A) and anti-carnosine (B). Arrow-heads indicate cells intensely labeled with anti-OMP but not anti-carnosine. Arrows indicate cells intensely labeled with anti-carnosine but not anti-OMP. Asterisks indicate cells that intensely labeled with both antibodies. The secondary antibodies used were FITC-labeled donkey anti-goat IgG and Texas Red-labeled donkey anti-rabbit IgG. (C)-(E) Anti-OMP- and control-treated cultures stained with peroxidase-labeled antibodies. (C) Numerous cells were labeled with anti-OMP. (D)-(E) Staining was not seen when normal goat serum was substituted for the primary antibody (D) or when the anti-OMP was pre-absorbed with purified OMP protein (E). Bar = 25 μ m for (A) and (B); bar = 25 μ m for (C)-(E).

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Unlike in tissue sections,² the overlap between carnosine-IR and OMP-IR was not complete in culture. We found all staining combinations [Fig. 2(A) and (B)]. The majority of cells, 74%, had intense carnosine-IR, with undetectable or very low OMP-IR (arrows). A smaller percentage, 22%, had intense immunoreactivity for both antibodies (asterisks), while even fewer, 4%, had intense OMP-IR with undetectable or very low carnosine-IR (arrow-heads) ($n = 1746$ cells counted, in two experiments). The percentage of carnosine-positive neurons that were OMP-negative appeared to be higher within the micro-noses (not quantified). Even given the limitations of staining techniques, the variations in intensity suggest that carnosine and OMP expression are not exclusively co-regulated, and that the culture conditions may affect this regulation in ways that are not observed *in vivo*.

Other cell types in micro-nose cultures

Olfactory nerve glia and CNS-derived astrocytes. The glial cells in the micro-nose cultures include both the type I astrocytes of the feeder layer and the olfactory nerve glia that were added with the olfactory/nasal cell suspension. The astrocyte feeder layer was over 90% positive when stained with antibodies to the glial fibrillary acidic protein, GFAP (not shown), which is a protein found exclusively in astrocytes *in vivo*.

Among the olfactory nerve glia, the Schwann cell-like glia stain very lightly with relatively high concentrations of anti-GFAP (1/1000 dilution) while the astrocyte-like glia (around 10% of all olfactory nerve glia) stain more densely with anti-GFAP.⁴³ Until immunoelectron microscopic studies are done to determine whether or not both types of glia ensheath ORN axons, we will assume that both types ensheath axons and both together comprise what other authors call the "olfactory ensheathing cells".¹²

The Schwann cell-like glia also stain intensely with antibodies to the S100 β protein, as described previously.⁴³ In the micro-nose cultures, where S100 β staining has not been described previously, the cells that showed S100 β -like immunoreactivity (S100-IR) were concentrated primarily in the nerve bundles interconnecting the micro-noses and formed also a network of positive fibers and cell bodies within micro-noses [Fig. 3(A)]. Individual olfactory Schwann cells were primarily bipolar, spindle-shaped cells with long thin processes [Fig. 3(A), arrows] that were similar to the cells that showed medium density staining with anti-NSE [compare arrows in Fig. 3(A) to Fig. 1(E) and (F), arrow-heads].

Nestin. All neuroepithelial cells in the early embryonic neural tube immunostain for the intermediate filament protein, nestin.¹⁵ This includes neuronal progenitor cells. Thus, antibodies to nestin have proven useful in identifying CNS neuronal progenitor cells in culture.⁵⁰ Later in embryonic development, nestin expression becomes restricted to the retained neuroepithelial cells (radial glia). After birth, nestin staining is detected primarily in Schwann cells.¹⁶ In the micro-nose cultures, cells that had nestin-like immunoreactivity were morphologically similar to cells that had S100-IR and they were present in the same patterns and amounts [cf. Fig. 3(A) and (B)]. No other cell types appeared to be stained.

CNPase. Antibodies to the oligodendrocyte-Schwann cell enzyme, 2'-3'-cyclic nucleotide 3'-

Fig. 3. Markers for other, non-neuronal, cell types in 15 day micro-nose cultures. (A) Anti-S100 β labeled Schwann cell-like olfactory nerve glia both inside micro-noses and on the feeder layer. Arrows point to nuclei of relatively isolated cells, to show cell shape. (B) Anti-nestin labeled cells very similar to those stained with anti-S100 β , if it is taken into account that nestin is an intracellular filament while S100 β is a cytoplasmic enzyme. (C) An antibody to the 200 kDa neurofilament protein (anti-NF) intensely labeled a minor number of cells that resembled olfactory nerve glia [arrows, compare to cells indicated with arrows in 3(A), 1(E) and 1(F)]. In addition, occasional fibers of unknown cell type with no visible cell nuclei were intensely labeled (not shown). (D) Staining with anti-CNPase labeled oligodendrocyte-like cells in cultures plated on feeder layers from high density cortical cells. These cells had small, round or triangular cell bodies and very branched cell processes (arrows). (E) Muscle cells were abundant in the cultures as shown by staining with an antibody specific for muscle actin. (F) Numerous macrophages (or microglia) were detected by staining with OX-42. (G) A clump of cartilage cells (arrow) was visible in an olfactory culture immunostained with anti-NST (DIC optics). (H) A better view of cartilage cells (a cluster is between the arrows) was provided by embedding fixed culture material in plastic, sectioning on an ultramicrotome at 2 μ m and staining with toluidine blue. The abundant extracellular matrix is evident. Two small micro-noses and their cavities (asterisks) are adjacent to the cartilage cluster. Bar = 80 μ m for (A)-(F); bar = 40 μ m for (G) and (H).

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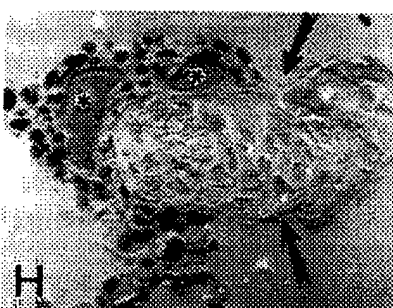
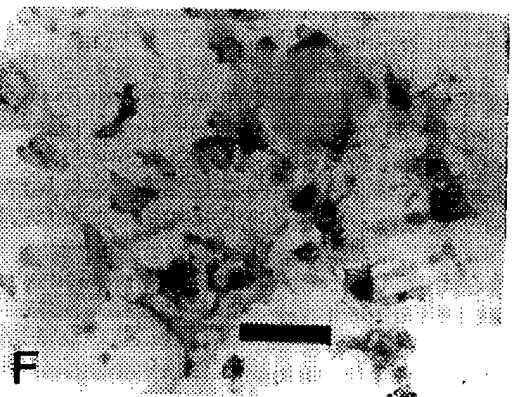
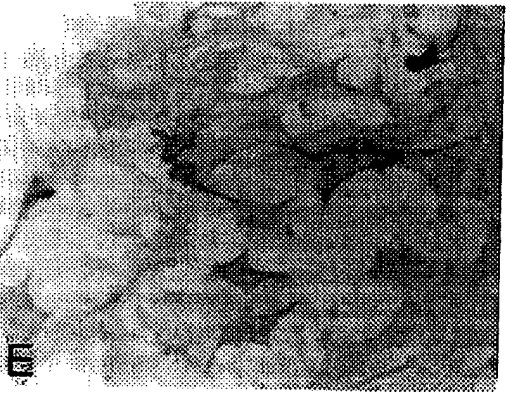
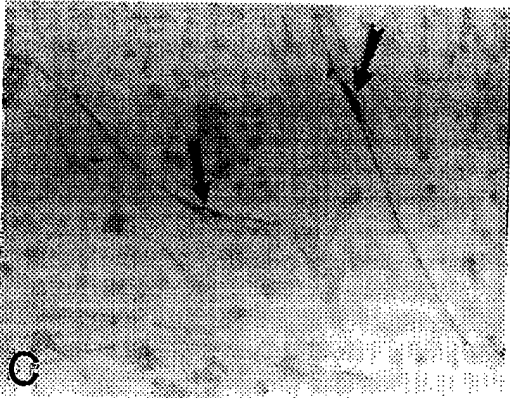
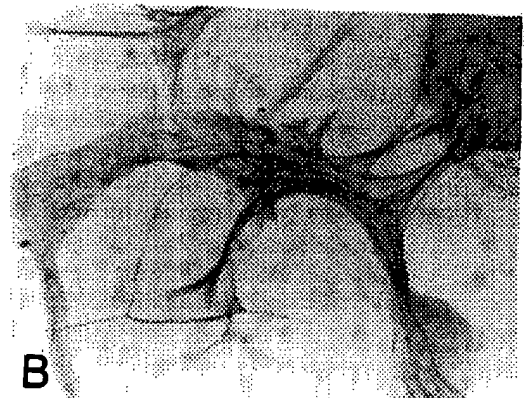
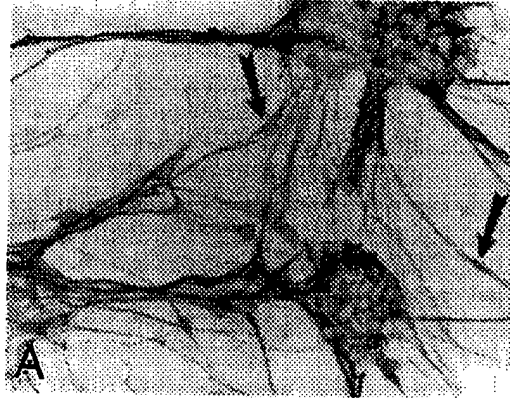
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phosphodiesterase (CNPase) specifically immunolabel oligodendrocytes, Schwann cells and the outer segments of retinal sensory cells *in situ*.⁶⁰ In olfactory cell cultures, positive cells were detected only when the feeder layer of cortical astrocytes was derived from high density cortical cell preparations. They were not detected when the astrocytes were from low density preparations. The difference is that the high density preparations contained multiple cell types in addition to astrocytes, while the low density preparations were almost pure astrocytes (with some muscle cells, see below). Cells with CNPase-IR were round with numerous thin, short, often extensively branched processes [Fig. 3(C), arrows]. They were scattered infrequently across the bed layer and on the outer surfaces of micro-noses; few were located within the micro-noses. There was no evidence that this antibody immunostained the Schwann cell-like olfactory glia, ORNs, or ORN dendrites or sensory cilia. These data suggest that the cells with CNPase-IR were oligodendrocytes derived from the astrocyte feeder layer.

Neurofilament protein. A monoclonal antibody to the 200 kDa neurofilament protein (NF) intensely labeled only a small number of cells and some fibers that lacked detectable cell bodies. When cell bodies could be detected, the cells morphologically resembled olfactory Schwann cells [Fig. 3(D), arrows, compare to individual glial cells in Fig. 3(A), arrows and Fig. 1(E) and (F), arrow-heads]. The numbers of both processes and cells were much lower than those detected with any of the neuronal or glial markers described here. Because of the small number of cells stained, the possibility that the majority were glia and because this antibody had a relatively high background (e.g. faint non-selective staining of all other cells in culture) this antibody was not considered useful for identification of any one cell type.

Muscle. A monoclonal antibody, HUC 1-1, that binds to an actin isoform found in smooth and skeletal, but not cardiovascular, muscle^{55,56} was used to determine muscle cell distribution in culture. Muscle cells were quite abundant in both the micro-nose cultures [Fig. 3(E)] and the astrocyte feeder layer (not shown). In tissue sections (not shown), muscle cells were abundant throughout the lamina propria. In particular, stained cells were present in the muscular walls of blood vessels, which suggests staining of smooth muscle, and around Bowman's glands, which suggests staining of myoepithelial cells.

Macrophages/microglia. The monoclonal antibody OX-42 recognizes the type 3 complement receptor and it binds to subsets of macrophages throughout the body and microglia in the CNS.²⁸ Numerous OX-42-positive cells were detected in the cultures, which suggests the presence of macrophages and/or microglia [Fig. 3(F)]. The OX-42-positive cells were large and round with no processes [Fig. 3(F)]. Positive cells were variably distributed across the cultures; some areas had patches of higher density, like the area shown in Fig. 3(F) while other areas had very few stained cells. The areas of high density were not always associated with micro-noses.

Cartilage. Clusters of cells that appeared to be chondrocytes secreting cartilage were occasionally detected in micro-nose cultures. These were noticeable with phase contrast optics, without immunostaining [Fig. 3(G)]. Cartilage-like cells were large, round and very homogeneous in size. The cells appeared to be surrounded with abundant extracellular material that was very bright in phase contrast optics [Fig. 3(G)]. The presence of abundant extracellular matrix deposition was more readily apparent once cultured material was embedded in plastic, sectioned and stained with toluidine blue [Fig. 3(H), the body of the cluster is between the arrows]. Also, we observed possible bone spicules in some plastic sections where it stained intensely pink with toluidine blue (not shown).

DISCUSSION

The main culture system described herein consisted of olfactory cells plated at relatively high density on astrocytes. This supported formation of "micro-noses" as well as abundant neuronal production and differentiation.⁴² Previous publications have described the basic structure of the micro-noses and the orientation of neurons within them.⁴⁶ In this report, we characterize further the neurons in the micro-nose cultures and continue examination of the many other cell types present in the cultures. These included olfactory Schwann cells, muscle cells, macrophages and cartilage cells. We do not describe localization and characterization of olfactory sustentacular cells

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(also known as the olfactory supporting cells), Bowman's gland cells or keratin-positive cells (which are possibly flat basal cells of the epithelium) although these cell types are also present in the micro-nose cultures as described in other publications^{45,47,48} (and Pixley, manuscript in preparation). This report, combined with our previous findings, represents the most complete characterization of any heterogeneous olfactory cell culture to date.

Neuronal markers

NST. Our preferred antibody marker for neuronal identification has been anti-NST, because NST antibodies gave dense staining of both immature (OMP-IR-negative) and mature (OMP-IR-positive) ORNs.⁴² Previously we compared a commercially available antibody to the monoclonal TUJ1 (a generous gift from A. Frankfurter) and found few differences.²⁵ Both NST antibodies gave robust staining at high dilutions (1/3000–1/10 000).

Antibodies to NST gave a faint staining of all other cells if high antibody concentrations were used ($< 1/500$ dilutions) (not shown). The TUJ1 antibody showed slightly less of this staining than the commercial antibody (data not shown). This faint staining was considered to be non-specific because it was uniform and on all non-neuronal cells; specific staining theoretically would be cell type-selective. Possible causes for this staining could be non-specific binding of antibody molecules or binding of impurities in the antibody solution. Impurities in the antibody solution are difficult to control for, and purified NST protein was not available, so we did not investigate the cause of the faint staining any further.

Faint non-neuronal staining could lead to inaccurate cell identification if neurons were not present in the cultures. In olfactory cell cultures grown without an astrocyte bed layer, the most abundant cell type is usually the olfactory Schwann cell, which morphologically resembles the ORNs. Faint staining of Schwann cells at high concentrations of anti-NST could appear to be neuronal staining if there were no neurons around for comparison.

N-CAM. Neuronal staining comparable to that detected with NST was observed with a commercial monoclonal antibody that recognized E-N-CAM and both the 140 and 180 forms of adult N-CAM (as per manufacturer's specifications). In tissue section studies, E-N-CAM is found on immature ORNs,³³ while the adult forms of N-CAM are found on both immature and mature ORNs.^{6,7,17,23,24,35,36}

Once again, faint staining of many cell types in the bed layer was detected at relatively high concentrations of the antibody (1/50 or 1/100 dilutions), ten times higher than that needed to reliably detect neuronal staining (1/500 dilution). Again, this staining did not appear to be cell type-specific, although we did not carefully examine the specificity.

Another group has reported N-CAM and E-N-CAM immunolabeling of astrocyte-like cells in dissociated cell cultures from mouse olfactory bulb tissues.³⁴ These bulb cell cultures are very similar to our astrocyte feeder layers, so some of our faint staining could be of the astrocytes in our cultures. Chuah and Au reported anti-N-CAM staining of purified ensheathing glia in culture using presumably the same antibody that we used (from the same commercial vendor).⁸ Thus the faint staining we observe could be of both astrocytes and olfactory Schwann cells.

In addition to glial cell staining, Chuah and colleagues later reported intense staining of ORNs in culture with the same anti-N-CAM.^{9,10} They concluded that this antibody was a selective marker for olfactory neurons when used at an appropriate dilution.^{9,10} Our results agree with these studies; anti-N-CAM gave intense staining of neurons at a wide range of concentrations and only this additional staining at very high concentrations. Because the neuronal staining was so much more intense, the antibody was a good marker for neurons. However, it is important, as was mentioned above, to be aware of the possibility of additional staining of glial cells and other cell types present in the cultures.

L1. In a previous study of mouse olfactory area tissue sections, L1-like immunoreactivity (L1-IR) was found in the olfactory nerve bundles on contact points between ORN axons and olfactory ensheathing cells and on contact points between ensheathing cells.^{33,36} These data suggest that L1 is produced by or associated with both ORNs and olfactory nerve glia. However, other researchers have not detected L1-IR on olfactory nerve glia.¹⁷ In confocal analyses of NST/L1 double labeling, we confirmed that, for our anti-L1, the staining was on both NST-positive and NST-negative fibers

(not shown). Further analysis is needed to determine if the additional staining is due to glial cell staining. What we can say is that LI-IR in our cultures is not solely selective for neurons.

NSE. As NSE is a highly soluble protein, special fixatives are needed for optimal detection.⁵⁷ With more commonly used fixatives, e.g. 4% paraformaldehyde, neuronal staining in olfactory cell cultures could not be distinguished from the background staining of practically all other cells present (Pixley, unpublished observation). As we show here, even with the specific fixative, care had to be taken to find an appropriate dilution that gave specific neuronal staining. The only cells that never exhibited staining were flattened epithelioid-like cells.

One possible explanation for the three levels of staining (none, light and intense) is expression of the three forms of the enolase. In other words, neurons and the unstained epithelioid cells may contain the γ_2 and α_2 homodimers, respectively, and the lightly stained cells could be expressing the $\alpha\gamma$ heterodimer. However, at present, we have no data supporting this suggestion.

In the micro-nose cultures, only a subset of neurons was stained with anti-NSE, based on comparisons to NST and N-CAM staining. An interesting possibility is that the NSE staining is functionally significant. In the CNS, neuronal progenitor cells switch from expression of non-neuronal enolase to NSE when they terminally differentiate.⁵⁸ In cultured dorsal root ganglion neurons from embryonic mice, intense staining for NSE is observed only in ganglion cells that are spontaneously active.⁵⁷ Thus, it might be of interest in the future to combine NSE staining with a functional assay to determine if only the NSE-positive neurons are spontaneously active.

Neurofilaments. The NF expression by ORNs *in vivo* (in tissue sections) is unlike that in other nervous tissues. Polyclonal antibodies that bind to all three NF proteins stain only around 0.1% of all ORNs in tissue sections.⁵⁹ However, a monoclonal antibody specific for the 200 kDa (heavy) NF protein stains a higher proportion of ORNs.⁵ In culture, Chuah *et al.*,¹⁰ found significant numbers of neurons stained with a monoclonal antibody specific for the heavy NF protein. However, they reported also that anti-NSE¹⁰ and anti-N-CAM⁹ stained greater numbers of neurons in similar cultures. This suggests that the anti-NF was not labeling all neurons.

In our cultures, if the densely labeled processes without identifiable cell bodies belonged to neurons, then our results agree with those of Chuah and colleagues that antibodies to the heavy NF protein do not immunolabel all neurons in culture. However, we did not determine the nature of these fibers, because they were very infrequent. This, plus the labeling of only a subset of possible olfactory glial cells, meant that antibodies to NF were not useful as cell type-specific markers in these cultures.

Olfactory marker protein. Olfactory marker protein is a soluble, 20 kDa cytoplasmic protein of unknown function that appears to be expressed only by mature ORNs *in vivo*, based on morphological and tritiated thymidine studies.^{13,14,20,30,37,38} In contrast, OMP is not expressed by a subset of neurons that have more immature characteristics, including their location in the epithelium and their expression of proteins characteristic of developing neurons.^{14,38} Thus, OMP expression is a useful marker for ORN differentiation and maturation.

The presence in the cultures of ORNs expressing OMP suggests that the cultures are sufficiently representative of the normal tissue that the ORNs can progress to this stage of differentiation. The accessibility of the ORNs in culture will allow us to more completely explore the idea that OMP expression is indeed a late stage in the differentiation of ORNs *in vitro*, as has been suggested by our initial tritiated thymidine studies *in vitro*.⁴² Furthermore, the cellular accessibility and control over the cellular environment will allow us to investigate the regulation of OMP expression.

Carnosine. Early studies proposed that carnosine was an ORN neurotransmitter, but more recent suggestions are that it is a neuromodulator; it does not fit all criteria of a neurotransmitter.^{2,31,54} Our demonstration of double labeling with anti-carnosine and anti-OMP is consistent with previous studies, both *in vivo*,^{2,51-53} and *in vitro*.⁴⁰ However, our lack of astrocyte staining is unusual because cortical astrocytes stain for carnosine in tissue sections from rats and mice.² We have no explanation at present for this lack of astrocytic staining.

An unusual finding was the incomplete overlap between carnosine and OMP staining in the olfactory cell cultures. This is surprising because, in tissue sections, the staining patterns are identical.^{2,51,52,53} However, our results concur with those of another cell culture study.⁴⁰ The most probable explanation is that the regulation of expression of the two compounds differs and that culture conditions differentially affect the expression of the two compounds. This is not hard to

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imagine, as OMP, which is a small protein, could be regulated by gene expression, while the dipeptide, carnosine, could be regulated by synthetic enzymes.

The two culture systems that have reported co-localization of OMP and carnosine staining described different proportions of overlap. Perroteau *et al.*,⁴⁰ reported that 50% of all OMP-positive neurons were carnosine-negative and 20% of all carnosine-positive neurons were OMP-negative. Our results showed percentages of 15 and 77%, respectively. These differences could be due to differences in the culture systems. Perroteau *et al.*⁴⁰ used dissociated olfactory cells from embryonic mouse tissues and their growth medium and substrate conditions differed from those used in our micro-nose cultures. Each of these variables (age of tissue, growth medium and substrate) can affect cellular differentiation, as has been demonstrated in other culture systems. Thus, they could differentially affect expression of carnosine and OMP.

Before we attempt to investigate the meaning of the co-expression of OMP and carnosine, or the lack thereof, we may need to explore one possible source of artifact in the staining. It was somewhat troubling that both antibodies gave nuclear staining when both of these highly soluble compounds are found in the cytoplasm. Fixation could affect differentially the nuclear localization of these compounds. This was found to be the case for NSE, another highly soluble, cytoplasmic compound. In the studies that identified the NSE fixative, the use of an inappropriate fixative gave staining of few cultured neurons and nuclear staining.⁵⁷ Use of a fixative designed to retard solubility of NSE resulted in higher counts of stained neurons and cytoplasmic staining. Thus, with another fixative, there may not be a lack of overlap between carnosine and OMP staining. However, in preliminary studies with a variety of fixatives including the NSE fixative, we have not noted any improvement in the cytoplasmic-to-nuclear staining ratios for either antibody. Further efforts are needed to address this question properly.

Controls for immunostaining

The controls used for polyclonal animal antisera were substitution of normal sera for the primary antibody (if pre-immune sera were not available) and dilution curves of the sera. A background problem that was relatively unique to culture was that normal sera gave significant background staining of small round cells with processes. This staining was similar to neuronal or glial staining, although it probably included staining of both, as well as macrophage staining. Background staining was reduced only by lowering the concentration of antisera. This meant that difficulties were encountered if polyclonal animal antisera did not give adequate signal-to-noise ratios at dilutions of lower than 1/1000 with the ABC-peroxidase labeling techniques (none of those illustrated here). With a low signal-to-noise antiserum, it was necessary to pre-absorb with the appropriate antigen to prove specificity. Most of the monoclonal antibodies and affinity-purified serum antibodies that we have tested to date have not given similar background problems.

Markers for other cell types

Olfactory nerve glia. The olfactory nerve glia, particularly the Schwann cell-like glia, have strong morphological similarities to ORNs and, as discussed above, they are labeled by some of the antibodies that also mark neurons. Thus, olfactory nerve glia could be mis-identified as olfactory neurons. In addition to this problem, there is no one antibody marker that intensely labels all the glial cell types. Anti-GFAP robustly labels CNS astrocytes and the few astrocyte-like olfactory glia, but does not readily or intensely label the large number of Schwann cell-like olfactory glia.⁴¹ Antibodies to S100 β and to the low-affinity nerve growth factor are good markers for the Schwann cell-like olfactory glia, but they do not label the astrocyte-like olfactory glia.⁴¹ Anti-nestin stained the Schwann cell-like glia strongly, but did not label the astrocyte bed layer. We have not yet determined if anti-nestin also stains astrocyte-like olfactory nerve glia. Thus, identification of all glial cells in olfactory cell cultures, with or without an astrocyte feeder layer, requires use of more than one marking antibody.

Nestin. With antibodies to the intermediate filament protein nestin, we expected to see staining of the olfactory neuronal progenitor cells, based on nestin staining of CNS neuronal stem cells in culture²⁶ and in immortalized cell lines.¹⁵ The lack of staining within the micro-noses and the fact

that all other staining was consistent with glial cell staining strongly suggests that nestin is not expressed by either the ORNs or the olfactory neuronal progenitor cells.

In tissue sections, anti-nestin staining was amorphous, although somewhat denser over the epithelium (not shown). Densely labeled nerve fibers in the lamina propria appeared to be trigeminal nerve fibers based on their position, frequency and the fact that they appeared to send single fibers up from the lamina propria into the epithelium where they ended above the basal lamina but below the apical surface of the epithelium (not shown). Our data are consistent with a recent *in situ* hybridization study that demonstrated no detectable expression of nestin mRNA in the embryonic or newborn mouse olfactory epithelium.¹¹ In contrast, anti-nestin staining was seen in basal cells in the vomeronasal epithelium.³⁹

Even if anti-nestin labels an as yet undetected population of olfactory neuronal progenitor cells in the cultures, anti-nestin would not be an optimal marker for cultured olfactory neuronal progenitor cells because of the much more extensive glial cell staining.

Muscle cells. Numerous muscle cells were identified in the cultures by use of a monoclonal antibody, HUC 1-1, that specifically binds to muscle actin. The HUC 1-1 antibody does not differentiate between smooth or striated muscle.^{55,56} The muscle cells detected in the astrocyte feeder layer were probably smooth muscle cells derived from blood vessel walls. Muscle cells in the olfactory cell mixture could come from blood vessels and from myoepithelial cells around Bowman's glands in the lamina propria. They could come also from the nasal swell bodies.³ The abundance of the muscle cells raises the possibility that they could exert some influence over ORN survival or differentiation *in vitro*.

Macrophages/microglia. Macrophages or microglial cells derived from the astrocyte feeder layer were abundant in the cultures. These are important to consider in studies of neuronal growth and survival because they are capable of producing numerous cytokines that can have either neurotrophic or neurotoxic actions.³²

Cartilage. The presence of clusters of cells that appeared to be cartilage cells suggests that removal of the lamina propria from the turbinate and septal cartilages also removes some chondroblasts. The appearance of bone spicules in the culture probably is due to differentiation of the chondrocytes, as efforts were made to remove all hard tissues prior to tissue dissociation and because, in the neonatal rat, the turbinates and septum are almost completely cartilaginous.

Interestingly, the presence of cartilage was not detrimental to neuronal growth. As shown in Fig. 3(E) and (F), neurons grew close to and indeed seemed to be tightly associated with at least some of the cartilage clusters. Chondrocytes, like muscle cells, are known to secrete numerous growth factors,²² some of which could be trophic for ORNs.

Oligodendrocytes. The oligodendrocyte-Schwann cell enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (E.C. 3. 1.4.37; CNPase) hydrolyzes 2',3'-cyclic nucleotides.⁶⁰ The CNPase is distinct from cyclic AMP (cAMP) phosphodiesterase, which hydrolyzes 3',5'-cyclic nucleotides. The function of CNPase is unknown, although its localization in cells that myelinate suggests that it may play a role in myelination.⁶⁰ The CNPase also is found in the retina associated with the inner and outer segments of both rod and cone cells, where it may function as a G protein.⁶⁰ In our cultures, neither olfactory glia nor ORNs were stained with either polyclonal or monoclonal antibodies to CNPase, as confirmed by both light and confocal microscopy (not all conditions are shown). The only staining appeared to be that of CNS oligodendrocytes that entered the cultures with the cortical feeder layer.

CONCLUSION

Olfactory cell cultures maintained differentiation of several divergent cell types. The cultures contained (and generate)⁴² both immature (NST, NSE, and N-CAM-positive) and mature (NST, OMP and carnosine-positive) ORNs. In addition, numerous other cell types showed robust survival and maintained or re-established relatively normal differentiation in the cultures. The cells thought to be of nasal origin included olfactory nerve glia, muscle cells, macrophages/microglia and cartilage

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(and possibly bone). We have previously described the presence of sustentacular cells and Bowman's gland cells in the cultures.^{45,47,48} Thus, we demonstrate, with this and other reports, what is probably the most extensive characterization to date of such heterogeneous olfactory cell cultures.

Our findings also demonstrate that this type of dissociated olfactory cell culture can maintain and support differentiation of most of the cell types found in the intact tissue. These cultures are interesting representations of the intact tissue, because they recreate, after plating, cell-cell interactions and relationships that are phenotypically similar to phenomenon normally observed *in situ*. As the cultures allow better access for experimental manipulation and better control of the environment than is possible *in situ*, we can now use these cultures to explore the regulation of neuronal differentiation, which may include extensive interactions with some or all of these other cell types.

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